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Chromium(VI) Bioremoval by *Pseudomonas* Bacteria: Role of Microbial Exudates for Natural Attenuation and Biotreatment of Cr(VI) Contamination

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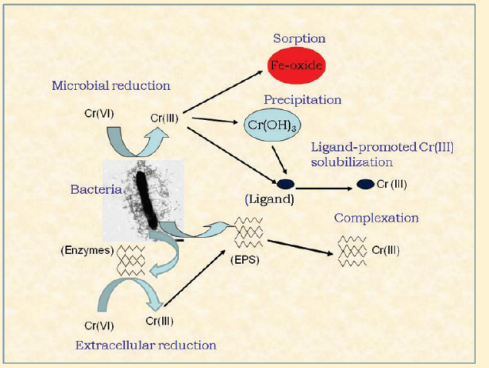
Supporting Information

ABSTRACT: Laboratory batch and column experiments were conducted to investigate the role of microbial exudates, e.g., exopolymeric substance (EPS) and alginic acid, on microbial Cr(VI) reduction by two different *Pseudomonas* strains (*P. putida* P18 and *P. aeuroginosa* P16) as a method for treating subsurface environment contaminated with Cr(VI). Our results indicate that microbial exudates significantly enhanced microbial Cr(VI) reduction rates by forming less toxic and highly soluble organo-Cr(III) complexes despite the fact Cr(III) has a very low solubility under the experimental conditions studied (e.g., pH 7). The formation of soluble organo-Cr(III) complexes led to the protection of the cells and chromate reductases from inactivation. In systems with no organic ligands, soluble organo-Cr(III) end products were formed between Cr(III) and the EPS directly released by bacteria due to cell lysis. Our results also provide evidence that cell lysis played an important role in microbial Cr(VI) reduction by *Pseudomonas* bacteria due to the release of constitutive reductases that intracellularly and/or extracellularly catalyzed the reduction of Cr(VI) to Cr(III). The overall results highlight the need for incorporation of the release and formation of organo-Cr(III) complexes into reactive transport models to more accurately design and monitor in situ microbial remediation techniques for the treatment of subsurface systems contaminated with Cr(VI).



INTRODUCTION

Chromium is an important industrial pollutant that may occur in the environment at elevated concentrations due to its extensive use in various industries including leather tanning, stainless-steel production, and electroplating.[1] Although chromium can exist in oxidation states ranging from Cr(-II) to Cr(VI), Cr(III) and Cr(VI) are the most dominant oxidation states in natural systems. While chromium(VI) is highly toxic to all forms of living organisms, Cr(III) has very low toxicity, and is immobile under slightly acidic to alkaline pH conditions due to its low solubility and highly sorptive characteristics.[1−3]

The mobility and toxicity of Cr(VI) in the subsurface environment can be reduced by converting it to Cr(III) using bacteria e.g., *Pseudomonas*.[4−11] Microbial Cr(VI) reduction has been shown to be an enzymatically catalyzed reaction attributed to soluble proteins for some bacteria[6] or cell membranes localized to the cytoplasm or periplasm for others (e.g., refs 11−14). For example, chromate reductases isolated from *P. putida*,[6,7,10] *Pseudomonas* sp. G1DM21,[11] and *Pseudomonas ambigua*[4] have been observed to be responsible for rapid Cr(VI) reduction to Cr(III).

A number of recent studies suggest that Cr(VI) reduction by some bacterial strains (e.g., *Pseudomonas* sp. G1DM21 and *P. putida*) led to the production of soluble Cr(III) end products and not $Cr(OH)_3$ precipitates (e.g., refs 11,13,15−17). The solubility of Cr(III) species can be significantly enhanced by strong interaction of Cr(III) with microbial exudates (e.g., exopolymeric substance (EPS), citrate, alginate) in natural systems.[3,10,13,18] For example, a study by Puzon et al.[19] suggested that an intracellularly located *E. coli* enzyme system converted Cr(VI) to a soluble and stable NAD^+-Cr(III) complex, and cytochrome c-mediated Cr(VI) reduction produces cytochrome c-Cr(III) adducts. Similarly, Puzon et al.[13] found that Cr(VI) reduction in the presence of cellular organic metabolites (e.g., citrate, ascorbate,

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malate) formed highly soluble organo-Cr(III) complexes, which are very stable over a broad pH range. Priester et al.[10] found that Cr(III) that formed after microbial reduction with *P. putida* was partly associated with exoploymeric substances (EPS).

Microbial exudates, e.g., EPS, are produced by microbes for a variety of purposes in response to environmental stresses (e.g., refs 10,18,20,21). Quantity and composition of EPS have been shown to vary depending upon bacterial strain and metal exposure in several studies.[10,18] For example, in a number of studies involving *P. putida* and *P. aeruginosa* strains, toxic substances such as Cr(VI) were found to be responsible for the enhanced production of microbial EPS (e.g., refs 10,21). Similarly, Sheng et al.[20] found that Cr(VI) stimulated the production of microbial EPS by the hydrogen-producing photosynthetic bacteria strain *Rhodopseudomonas acidophila*. In anaerobic chemostats fed on glucose, Aquino and Stuckey[18] observed that exposure to excess Cr(VI) led to an enhanced EPS production.

Despite the overwhelming evidence on the production of EPS by microbes during microbial Cr(VI) reduction, little is known about Cr−EPS interactions. Specifically, this study was conducted to determine whether microbial exudates released by bacteria play any role in microbial Cr(VI) reduction by two different commonly occurring soil microorganisms *Pseudmonas putida* P18 *and Pseudomonas aeruginosa* P16. The microbial exudates used in the study include bacterial exopolymeric substances (EPS) directly extracted from *P. aeruginosa* and *P. putida*, galacturonic, glucuronic, and alginic acids (AA). Here, alginic, galacturonic, and glucuronic acids, uronic acids identified as a main constituent of microbial EPS, were used as model organic ligands with known chemical structures.[22] Representative chemical structures of galacturonic, glucuronic, and alginic acids can be found in Figure S1 (Supporting Information (SI)). Uronic acids are continuously produced by some soil bacteria in subsurface systems. For example, bacterially produced alginate is commonly present in subsurface systems due to its production by N_2-fixing bacteria of the genus *Azobacter* and *P. aeruginosa*.[23] Alginic acid is an unbranched, binary copolymer composed of 1,4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues arranged in a nonregular blockwise order along the chain.[3] Our previous studies show that Cr(III) forms relatively strong complexes with microbial EPS, galacturonic, glucuronic, and alginic acids.[3,24]

EXPERIMENTAL SECTION

Materials. Unless stated otherwise, all chemicals used in the experiments were reagent grade or better. Water for all experiments was supplied from a Millipore (Simplicity 185) ultraviolet (UV)-water system. Cr (VI) stock solution was prepared by dissolving potassium dichromate ($K_2Cr_2O_7$) (Merck) in UV-water. Similarly, chromium(III)-nitrate-nonahydrate (Merck) was used as the source for Cr(III) in all experiments. A 200 mg portion of alginic acid purchased as alginic acid sodium monohydrate (Aldrich) was dissolved in 50 mL of UV-water, and its pH was adjusted to ~pH 7 with sodium hydroxide. Stock solutions of D(+)-glucuronic acid sodium salt monohydrate ($C_6H_9NaO_7 \cdot H_2O$) (Merck) and D(+) galacturonic acid ($C_6H_{10}O_7 \cdot H_2O$) (Fluka) were also prepared in UV water, and used in the experiments. All stock solutions were stored in amber glass bottles in the dark at 4 °C.

Isolation and purification of bacterial EPS were done as described by Hung et al.[25] Detailed information regarding EPS isolation and purification from bacteria can be found in SI page S3.

Preparation of Media and Growth Conditions. The medium for Cr (VI) reduction experiments (TSB) for *P. putida* P18 consisted of peptone from casein (17 g), peptone from soy meal (3 g), glucose (2.5 g) NaCl (5 g), and dipotassium hydrogen phosphate (2.5 g), and the medium (Luria−Bartani; LB) for *P. aeruginosa* P16 contained tryptone (10 g), yeast extract (5 g), and NaCl (10 g). The activated cultures inoculated in growth media were aerobically incubated at 30 °C for *P. putida* and 37 °C for *P. aeruginosa* P16 with constant shaking at 100 rpm and culture growth was monitored by measuring optical density (OD) at 600 nm. The culture suspensions were prepared and adjusted by comparing against 0.5 McFarland turbidity standard tubes (1.5×10^8 cfu/mL).

Cr (VI) Reduction and Biosorption Experiments. Kinetic experiments were performed to determine the microbial Cr(VI) reduction rates by *P. putida* P18 and *P. aeruginosa* P16 in the absence or presence of complexing ligands. The 250-mL flasks containing 100 mL of media with a desired Cr (VI) and complexing ligand concentration were inoculated with 2 mL of cultures at logarithmic phase. The initial concentrations of Cr-(VI) used in the experiments were 0.337 mM and 2.5 mM for *P. putida* P18 and *P. aeruginosa* P16, respectively. These concentrations were used in the experiments based on information gathered from minimum inhibitory concentration (MIC) tests (Supporting Information page S3). The initial pH of the media was buffered to 7.0 ± 0.1 using an appropriate amount of $NaHCO_3$ (0.088 mM). All media were autoclaved at 121 °C for 15 min before use in microbial Cr(VI) reduction experiments. In experiments involving complexing agents, the media contained 1 g L^{-1} galacturonic acid, glucuronic acid, alginic acid, and microbial EPSs directly extracted from *P. putida* P18 and *P. aeruginosa* P16 strains. Cultures were then aerobically incubated at 37 °C with constant shaking at 200 rpm. Controls composed of 100 mL of media with complexing ligands were also prepared to monitor for abiotic Cr(VI) reduction. Immediately after inoculation with bacteria, samples were drawn at regular time intervals (every 6 h) and centrifuged at 10 000 rpm for 15 min. The concentration of Cr (VI) in the supernatant was determined colorimetrically at 540 nm by UV spectrophotometer using diphenylcarbazide reagent.[26] Total chromium content of samples was determined using ICP-MS (Agilent 7500 ce) with a detection limit of 4×10^{-10} M. Cr(III) was calculated from the difference between total chromium and Cr(VI). The growth of cells was also routinely monitored by measuring optical density (OD) at 600 nm. The experiments were carried out in duplicate.

Additional kinetic experiments were also performed to determine the role of initial Cr(III) concentration on Cr (VI) reduction by bacteria in systems containing alginic acid or not. Cr (III) stock solution was filter-sterilized prior to use. Then, a desired amount of Cr(III) was added to 100 mL of growth medium containing 0.088 mM $NaHCO_3$ and a desired concentration of Cr(VI) at pH 7 (e.g., 0.096 mM Cr (III) and 0.24 mM Cr(VI) for *P. putida* P18 and 0.577 mM Cr(III) and 1.92 mM Cr(VI) for *P. aeruginosa* P16). Experimental conditions for batch kinetic bioreduction experiments are presented in Table 1.

To determine the extent of Cr(VI) biosorption onto cell material, the heat killed cells (~ 1 g dry weight) were added to 100 mL of chromium solution. The samples were shaken at 100 rpm. The Cr (VI) concentration before adding the biomass as well as at different times of incubation was determined by diphenylcarbazide method. The experiments were carried out in duplicate.

X-ray Near Edge Spectroscopy (XANES). XANES analysis was conducted to determine the oxidation state of Cr on cells exposed to a desired Cr(VI) concentration in nutrient media. The cells were then centrifuged at 4000 rpm, and prepared for

Table 1. Experimental Conditions for Batch Reduction Experiments and First Order Model Kinetic Coefficients

				k^a (L·mg^{-1} [dry wt] of cell·h^{-1})	
culture	ligand	initial Cr(VI) concn. (mM)	initial Cr(III) concn. (mM)	initial stage	final stage
P. putida P18[b]		0.337		0.052	0.052
	galacturonic acid	0.337		0.075	0.195
	glucuronic acid	0.337		0.051	0.289
	alginic acid	0.337		0.026	0.138
	P18 EPS	0.337		0.083	0.083
	alginic acid	0.24	0.096	0.044	0.141
		0.24	0.096	0.019	0.173
	alginic acid	0.24		0.131	0.131
		0.24		0.056	0.056
P. aeruginosa P16[c]		2.5		0.017	0.017
	galacturonic acid	2.5		0.020	0.02
	glucuronic acid	2.5		0.037	0.037
	alginic acid	2.5		0.022	0.022
	P16 EPS	2.5		0.028	0.071
	alginic acid	1.92	0.577	0.013	0.229
		1.92	0.577	0.016	0.016
	alginic acid	1.92		0.038	0.598
		1.92		0.032	0.343

[a] In all models, correlation coefficient (R^2) > 0.95. [b] Initial cell concentration = 0. 96 mg [dry wt] mL^{-1}. [c] Initial cell concentration = 0.49 mg [dry wt] mL^{-1}.

analysis with XANES. The XANES analysis was performed at the Cr K edge (5989 eV) on beamline X10C at the National Synchrotron Light Source (NSLS) in the fluorescence mode using a 13 element Ge detector. Samples were placed on an Al sample holder having cutout geometry of mm (H) × 20 mm (L) × 1.5 mm thickness and sealed with Kapton tape. Standards included Cr^{6+} [potassium chromate, K_2CrO_4] and Cr^{3+} [chromium hydroxide, $Cr(OH)_3$]. Spectra (3 scans per sample) were collected from 200 eV below the absorption edge to 300 eV above the absorption edge. Data in the XANES region was collected with an energy step of 0.5 eV at 2.0 s per interval. A chromium metal foil was placed in the reference channel and was run simultaneously with each sample to monitor shifts in the beamline energy. XANES analysis, which included background subtraction and normalization of the signal to the edge-jump, was performed using ATHENA and AUTOBACK analysis software.[27] The position of the first derivative of the absorption edge energy was used to determine the oxidation state.

Column Experiments. Column experiments were performed to study the effects of microbial exudates on microbial Cr(VI) reduction under advective conditions similar to those that might be observed in permeable reactive treatment barriers (PRB) in the field. The laboratory column studies were conducted using liquid chromatography columns with Teflon fittings with an inner diameter of 2.2 cm. A reciprocating dual-piston high-performance liquid chromatography pump (Alltech model 301 HPLC Pump with inert PEEK heads) was used to deliver solutions. Samples were collected and analyzed for Cr contents after elution through the column using an ISCO Instruments Retriever II Fraction Collector. All column experiments were conducted at 22 ± 2 °C and a flow rate of 0.03 mL min^{-1}. Experimental conditions for all column experiments are presented in Table S1 (Supporting Information).

Duplicate column tests were performed with columns containing washed quartz sand (lower portion) and soil (upper portion). The length of the column packed with the quartz sand was 11.0 cm and that of soil was 1.5 cm (Supporting Information, Figure S2). Quartz sand was sieved to a size fraction of 75−425 μm, and treated to remove organic residuals and the suspended iron and aluminum oxides using the method described by Kantar and Honeyman.[28] The soil samples, collected from unpolluted agricultural fields located in Mersin, Turkey, were treated and used in the column experiments (see Supporting Information, pages S4−S6 for detailed information on soil treatment and properties).

Prior to performing experiments, the columns were continuously flushed with the growth medium (LB) with no Cr(VI) at pH 7 for 24 h to establish a steady-state flow in the columns. All solutions were prepared using UV-water with a pH of 7.0 and contained 0.088 mM $NaHCO_3$ to minimize equilibration times and pH drift. After equilibration, the Cr(VI) solutions containing a desired amount of Cr(VI) and/or organic ligand (e.g., alginic acid) at pH 7 and ionic strength of 0.001 M NaCl were pumped through the columns. To characterize the columns, bromide (Br^-) was injected as a conservative tracer (see Supporting Information for a detailed description, pages S6−S7).

Modeling of Cr (VI) Reduction. A first-order model was used to determine microbial Cr(VI) reduction kinetics in the absence and presence of complexing organic ligands:

$$\mathrm{Cr(VI)} = \mathrm{Cr(VI)_o} e^{-kXt} \quad (1)$$

where Cr(VI) is the model predicted concentration of chromium (M), $Cr(VI)_o$ is the initial Cr(VI) concentration, k is the first order rate constant, t is time (h), and X is the bacterial cell concentration in milligrams (dry weight) mL^{-1}. Equation 1 can be linearized by taking the natural logarithm of both sides as follows:

$$\ln[\mathrm{Cr(VI)}] = \ln[\mathrm{Cr(VI)_o}] - kXt \quad (2)$$

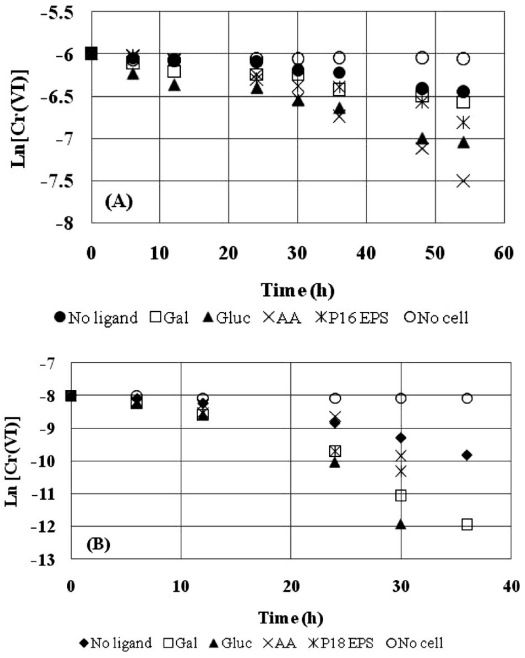

Figure 1. Whole cell reduction of Cr(VI) in the absence or presence of 1 g L^{-1} microbial exudates (galacturonic (Gal), glucuronic (Gluc), alginic (AA) acids, and microbial EPS) by (A) *P. aeruginosa* P16: Cr(VI) = 2.5 mM, and (B) *P. putida* P18: Cr(VI) = 0.337 mM. All solutions prepared in LB or TSB nutrient media were buffered to pH 7 with 0.088 mM $NaHCO_3$.

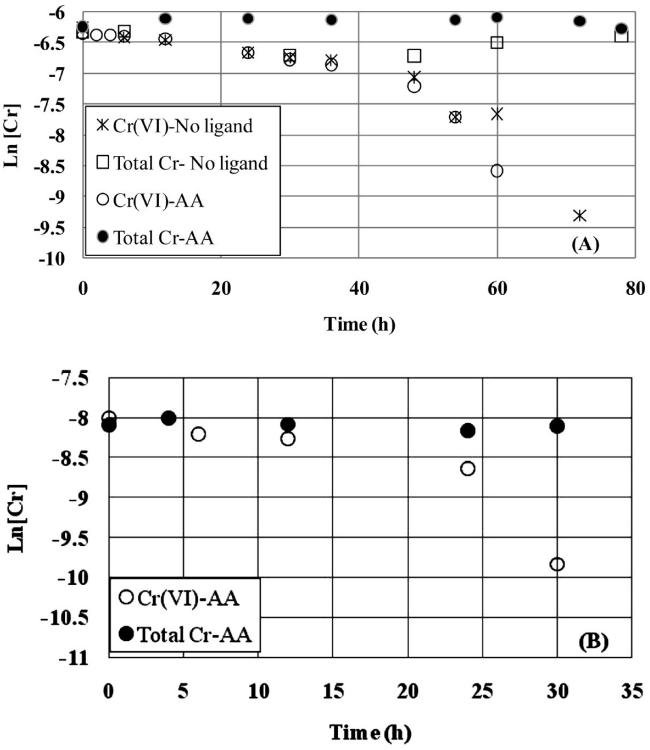

Figure 2. Whole cell reduction of Cr(VI) and total chromium (Total Cr) analysis in the absence or presence of 1 g L^{-1} alginic acid (AA) by (A) *P. aeruginosa* P16: Cr(VI) = 2.5 mM, and (B) *P. putida* P18: Cr(VI) = 0.337 mM. All solutions prepared in LB or TSB nutrient media were buffered to pH 7 with 0.088 mM $NaHCO_3$.

RESULTS

Effect of Complexing Ligands on Bacterial Chromium Reduction. The effects of complexing ligands such as galacturonic acid, glucuronic acid, alginic acid, and microbial EPS on Cr (VI) reduction by *P. putida* and *P. aeruginosa* were tested as given in Figure 1. Chromium(VI) reduction was observed in the presence of all four complexing ligands tested, although the extent of reduction varied from one ligand to another. For example, the strongest stimulatory effect on Cr(VI) reduction with *P. aeruginosa* was obtained with alginic acid, and decreased in the order of alginic acid > glucuronic acid > EPS > galacturoniac acid. Complexing ligands had similar stimulatory effects on Cr(VI) reduction with *P. putida* (Figure 1) with maximum effect observed in the presence of glucuronic and galacturonic acids. Controls without cells exhibited little or no Cr(VI) reduction in the absence and presence of organic ligands studied indicating that abiotic processes had no effect on overall reduction (Figure 1). Experiments with heat-inactivated cells indicate that biosorption onto cell material had a negligible impact for the loss of Cr(VI) from solution (Data not shown).

The mass balance analysis revealed that the Cr(VI) reduction by *Pseudomonas* bacteria led to the production of soluble Cr(III) end products and not the precipitation of Cr(III) as $Cr(OH)_{3(S)}$. In addition, the presence of complexing ligands increased Cr solubility (Figure 2). For *P. aeuroginosa* P 16, the amount of total Cr in solution decreased slightly in the early stages of Cr(VI) reduction (t < 40 h), then started to increase after 40 h indicating the formation of soluble Cr species in solution (Figure 2A). In experiments involving alginic acid, all of the chromium added remained in solution with no precipitation at any stages of microbial reduction. Similarly, microbial Cr(VI) reduction with *P. putida* produced soluble end-products, especially in the presence of complexing ligands such as alginic acid (Figure 2B). These results are in good agreement with the observations of Mabbett et al.,[29] Desai et al.,[11] Puzon et al.,[13] and Aquino and Stuckey.[18] For example, Mabbett et al.[29] reported on the reduction of Cr(VI) by *Desulfovibrio vulgaris* ATCC 29579 in anaerobic resting cell suspensions. Their results indicate that bioreduction occurred only in the presence of low molecular chelating agents (e.g., EDTA, citrate). Similarly, Puzon et al.[13] found that the biotic Cr(VI) reduction by *E. coli* (ATCC 11105) in the presence of cellular organic metabolites (e.g., citrate) formed both soluble and insoluble organo-Cr(III) end products. Desai et al.[11] observed an enhanced chromate reductase activity by *Pseudomonas* sp. G1DM21 in the presence of electron donors such as citrate, acetate, and succinate.

Chromium Oxidation State. XANES spectra for the standards and samples are presented in Figure 3. The first derivatives of the absorption edge energy for Cr(VI) and Cr(III) standards were found at 6005.6 and 6003.3 eV, respectively. In addition, the Cr^{6+} standard exhibited a pre-edge peak at 5993.3 eV due to the tetrahedral coordination (1s > 3d transition) of the chromate oxygen atoms. The dotted line in the plots is set to the absorption edge for $Cr(OH)_3$ at 6003.3 eV. The absorption edge energy for Cr associated with the pellets varied between 6003.3 and 6003.6 eV. In systems involving complexing ligands, the Cr absorption edge position appeared to vary slightly between 6003.3 and 6003.5 eV for the P16 and P18 samples (Figure 3A, B). This is within the range expected for the absorption edge position for the Cr(III) standard and the absence of the pre-edge peak confirms that the Cr is in the trivalent oxidation state.

Effect of Cr (III) on Reduction of Cr (VI). As indicated by the XANES analysis, Cr(III) was one of the end products of microbial Cr(VI) reduction. Thus, the effects of Cr(III) on Cr(VI) reduction were also evaluated as exhibited in Figure 4. It is clear that Cr (III)

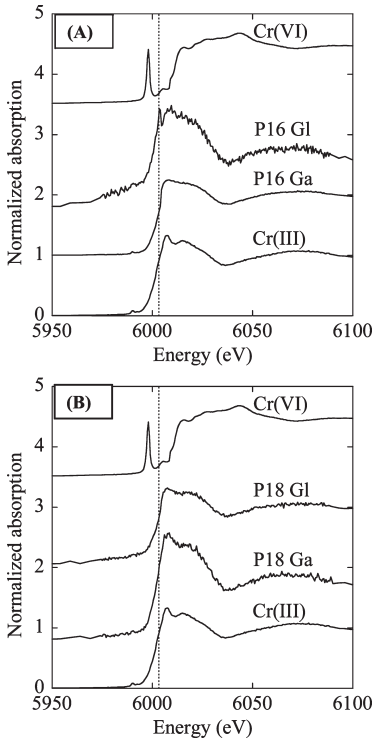

Figure 3. Comparison of absorption edge positions for Cr(III) and Cr(VI) standards and Cr-containing (A) *P. aeruginosa* P16 and (B) *P. putida* P18 sample pellets obtained under aerobic conditions in the presence of bacteria and 1 g L^{-1} glucuronic (Gl) and 1 g L^{-1} galacturonic (Ga) acids. The dotted line is set to the absorption edge for $Cr(OH)_3$ at 6003.3 eV. Whereas P16 cells were initially exposed to 2.5 mM Cr(VI), P18 cells were in contact with 0.577 mM Cr(VI).

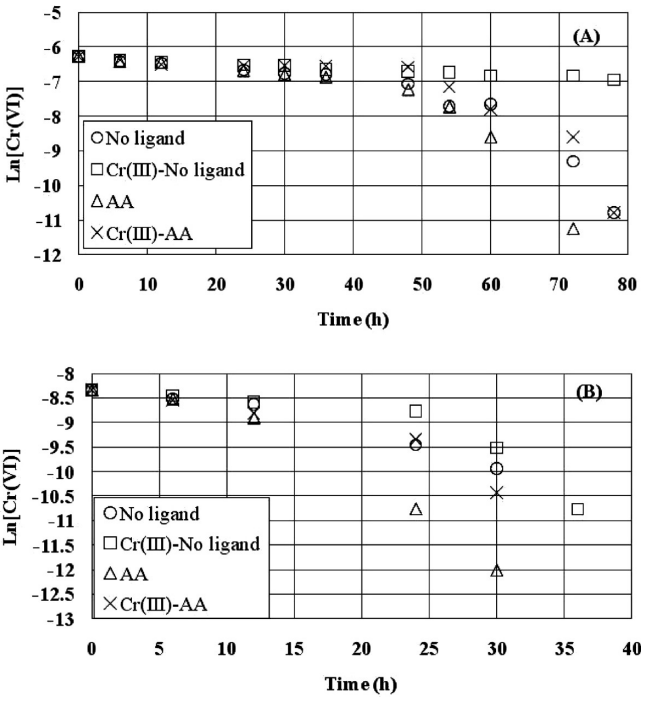

Figure 4. Effect of Cr(III) on whole cell reduction of Cr(VI) in the absence or presence of 1 g L^{-1} alginic acid (AA) by (A) *P. aeruginosa* P16: Cr(VI) = 1.92 mM, Cr(III) = 0.577 mM and (B) *P. putida* P18: Cr(VI) = 0.24 mM, Cr(III) = 0.096 mM. All solutions prepared in LB or TSB nutrient media were buffered to pH 7 with 0.088 mM $NaHCO_3$.

strongly inhibited the Cr(VI) reductase activities in both *P. aeruginosa* and *P.putida* with much smaller Cr(VI) reduction rates relative to non-Cr(III) containing systems. As indicated above, organic ligands displayed a stimulatory effect on microbial Cr(VI) reduction. Thus, a separate experiment was conducted to determine if organic acids stimulated microbial Cr(VI) reduction in systems initially containing Cr(III) along with Cr(VI). For example, in *P. aeruginosa*, the addition of alginic acid to systems containing 0.577 mM Cr(III) and 1.92 mM Cr(VI) led to an enhanced Cr(VI) reduction relative to nonalginic acid containing systems, probably due to the formation of less toxic Cr(III)-ligand complexes. A similar trend was observed in the cases of *P. putida* (Figure 4B).

An interesting aspect of microbial Cr(VI) reduction in absence or presence of complexing ligands is that the reaction kinetics do not strictly follow a first order pattern (Figure 4). In fact, nearly all of the curves have two distinct reaction stages. For example, except for the growth medium amended with only Cr(III), the Cr(VI) reduction by *P. aeruginosa* in the absence or presence of alginic acid exhibits a slower rate of initial reduction (t < 45 h) followed by a much faster rate in the final stage for 1.92 mM Cr(VI) (Figure 4A). In systems amended with alginic acid and Cr(III), while only 27% of total Cr(VI) added was reduced in the initial stage (t < 45 h), the remaining portion was completely reduced within less than 24 h in the second stage. On the other hand, for the growth medium amended with only alginic acid, 61% of the total Cr(VI) was reduced within the initial stage (<45 h). The experiments performed with *P. putida* exhibit similar reaction rates (Figure 4B). Kinetic rate constants for the initial and final stages are presented in Table 1. These results indicate that total chromium concentration and Cr(III)/ligand ratio have a pronounced impact on Cr(VI) reduction with *Pseudomonas* bacteria under the experimental conditions studied.

Column Experiments. Microbial exudates are ubiquitous in the subsurface environment and as such may play an important role in microbial Cr(VI) reduction and subsequent mobility or immobility in systems contaminated with chromium. Here, column experiments were performed to more accurately understand the effects of microbial exudates on Cr(VI) reduction in continuous flow bioreactors or PRBs. In these experiments, Cr(VI) (0.577 mM) and alginic acid were co-injected through the columns previously inoculated with *P. aeruginosa*. Figure S4 (Supporting Information) shows breakthrough curves for Cr(VI) at pH 7 and in the presence of increasing alginic acid concentration. Note that microbial Cr(VI) reduction was significantly enhanced in the columns in the presence of complexing ligands, e.g., microbial exudates, as was the case observed in batch microbial Cr(VI) reduction experiments (see Supporting Information for a detailed description, pages S9−S10).

DISCUSSION

Recently, organic ligands capable of reducing Cr(VI) have emerged as a reasonable means of enhancing or reducing Cr mobility and as a key factor to be addressed for assessing the long-term environmental stewardship of sites contaminated with chromium (e.g., ref 2). Several researchers have, for example, identified the potential role of microbial exudates in Cr oxide solubilization as a key question to be addressed for the development of remediation strategies and accurate assessments of environmental risks.[10,13,18,19] Although a number of studies show

that exposure to toxic substances such as Cr(VI) leads to the production of exopolymeric substances (EPS) and low molecular uronic acids (galacturonic, alginic, and glucuronic acids) by microorganisms, their role on microbial Cr(VI) reduction is not clearly established in the literature. As shown in Figure 1, organic ligands used played a stimulatory effect on the reduction of Cr(VI) to Cr(III) by *Pseudomonas* bacteria. According to Mabbett et al.[29] and Desai et al.,[11] this stimulatory effect was caused by the formation of less toxic soluble Cr(III)-ligand complexes:

$$\text{Ligand} + \text{Cr(VI)} \xrightarrow{\text{Cells/Enzymes}} \text{Cr(III)} \rightarrow \text{Cr(III)} - \text{Ligand} \quad (3)$$

Microbial EPS and low molecular uronic acids, e.g., galacturonic and glucuronic acids, contain several functional groups, e.g., carboxylic groups (COOH), available for complexation with metal ions such as Cr^{3+}. In our previous study, for instance, we found that the COOH groups played an important role in Cr(III) binding with galacturonic, glucuronic, and alginic acids.[3] Similarly, in a modeling study with EPS extracted from *P. putida* P18 and *P. aeruginosa*, we also determined that the acid/base properties of EPSs could be best characterized by invoking four different types of acid functional groups with arbitrarily assigned pK_a values of 4, 6, 8, and 10.[24] These functional groups can be operationally defined as carboxyl (HL_1), carboxyl/phosphoric (HL_2), phosphoric (HL_3), and hydroxyl/phenolic (HL_4) sites, respectively. Kantar et al.[24] also show that the main functional groups of EPS involved in Cr complexation were the HL_2 functional groups, indicating that both carboxyl and phosphoric acid sites contributed to Cr(III) binding by EPS (Table S2).

The complexation reactions between Cr(III) and microbial exudates (e.g., EPS) have a pronounced impact on Cr solubility, bioavailability, and sorption/transport behavior in subsurface systems.[3,10,13,30] For example, Figure S5 shows the effects of EPS on Cr(III) solubility as a function of pH (Supporting Information). Whereas in systems with no EPS, Cr(III) solubility decreases with increasing pH, and reaches a minimum at pH 7 due to the formation of $Cr(OH)_{3(s)}$, it remains soluble over a wide pH range in systems containing EPS even under alkaline pH conditions.

In general, the formation of Cr(III)-organic ligand complexes may affect microbial Cr(VI) reduction in two ways: protection of cells and enzymes responsible for Cr(VI) reduction. Figure 5 shows that alginic acid protected the cells from the toxic effect of chromium, and thus led to enhanced cell growth. Note that in systems with only Cr(III), there was no change in cell biomass up to 30 h of reaction time indicating that Cr(III) had an adverse effect on cell growth especially for *P. aeruginosa*. When Cr(III) was simultaneously added with alginic acid, this adverse effect diminished significantly. As indicated by Mabbett et al.,[29] Cr(III) complexed with organic ligands would not be in a form available for other molecules such as surface sites on microbial cells, responsible for microbial Cr(VI) reduction. As seen in Figure S6 (Supporting Information), the amount of Cr(III) biosorbed or precipitated onto P16 cells decreased with increasing alginic acid concentration under the experimental conditions studied (e.g., pH 7). In addition to cell protection, studies by Horitsu et al.,[4] Mabbett et al.,[29] and Desai et al.[11] show that low molecular weight organic molecules protected the chromate reductase

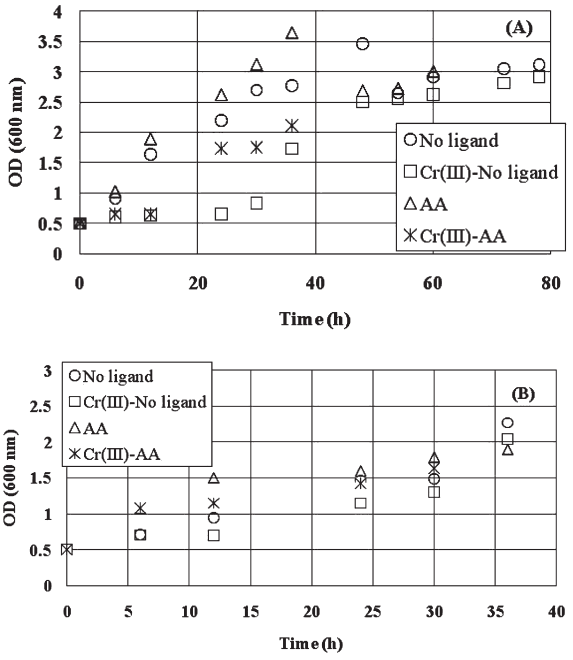

Figure 5. Effects of Cr(III) on cell growth in the absence or presence of 1 g L^{-1} alginic acid (AA) by (A) *P. aeruginosa* P16: Cr(VI) = 1.92 mM, Cr(III) = 0.577 mM and (B) *P. putida* P18: Cr(VI) = 0.24 mM, Cr(III) = 0.096 mM. All solutions prepared in LB or TSB nutrient media were buffered to pH 7 with 0.088 mM $NaHCO_3$. Corresponding Cr(VI) reduction results are given in Figure 4.

enzymes from inactivation by removing a toxic product of microbial reduction e.g., Cr(III) species, thereby protecting the cytoplasmic and/or periplasmic enzymes responsible for Cr(VI) reduction from chemical attack. Mabbett et al.[29] found a close connection between the rate of microbial Cr(VI) reduction and the strength of the ligand/chelate complex of Cr(III) in systems involving ligands/chelating agents such as citrate, EDTA and NTA. As noted in Figure 4, the microbial Cr(VI) reduction curves were described by two major stages. In the first stage, the reaction rate was found to be much slower than the rates observed in the final stage (Table 1). This indicates that chromium concentration and Cr(III)/ligand ratio are major controlling factors for microbial Cr(VI) reduction by *Pseudomonas* bacteria.

Mass balance analysis indicates that the reduced Cr forms soluble end-products even in the absence of any organic ligands, indicating that a soluble chromate reductase was responsible for Cr(VI) reduction. Note that in the absence of any organic ligand, the total Cr content in solution initially decreased with increasing time ($t < 40$ h) and then increased back to the total Cr concentration initially added to the system for *P. aeruginosa* P16 (Figure 2A). Whereas the decrease observed in total Cr content at $t < 40$ h may be explained through biosorption of Cr onto cell walls, the increase in Cr concentration in solution at $t > 40$ h is an indication of complex formation between Cr(III) and microbial EPS released from the bacteria due to cell lysis. It is possible that cell lysis led to the release of constitutive reductases that extracellularly catalyzed the reduction of Cr(VI) to Cr(III) as was the case for *P. putida*.[10] As given in Figure 5A, the cell growth increased with increasing time, and reached a maximum at $t = 40$ h, and decreased sharply until the constant growth conditions were established in the system. The decrease observed in the cell growth at $t = 40$ h coincides with the increase observed in total Cr

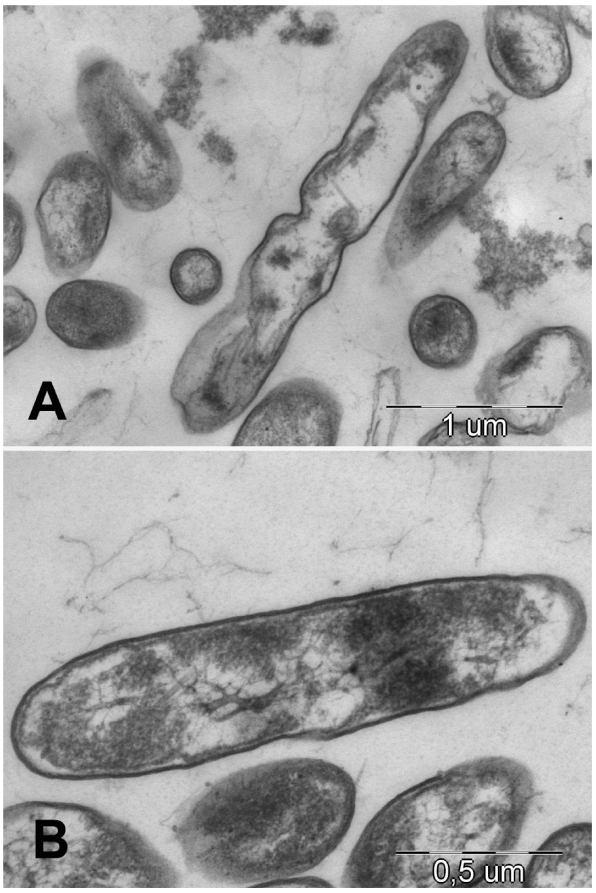

Figure 6. TEM images of *P. putida* P18 grown in TSB nutrient medium containing 1 g L^{-1} glucuronic acid with (A) 0.337 mM Cr(VI) and (B) no Cr(VI).

concentration as given in Figure 4A. These results indicate that regardless of where Cr(VI) reduction occurred, the cell lysis played an important role in Cr(VI) reduction. Ishibashi et al.[6] observed that chromate reductase activity was associated with soluble protein and not with the membrane fraction. Similarly, Priester et al.[10] found that cell lysis released constitutive reductases that catalyzed the extra cellular reduction of Cr(VI) to Cr (III) by *P. putida*. Desai et al.[11] and Park et al.[7] suggest that a soluble chromate reductase associated with the cytoplasmic membrane catalyzed Cr(VI) reduction by *Pseudomonas* sp. G1DM21 by transferring initial one electron to Cr(VI) to form an intermediate Cr(V), followed by two electron transfer for Cr(III) formation. McLean and Beveridge[8] report that the chromate reductases originated in the cytoplasm left cells by cell lysis, and reduced Cr(VI) extracellulary. On the other hand, according to Puzon et al.,[13] Cr(VI), intracellularly reduced in the cytoplasm by a bacterial enzyme, using NADH as the reductant, left cells by cell lysis leading to the formation of soluble Cr(III) end products in solution. Figure 6 shows transmission electron microscopy (TEM) images of *P. putida* P18 cells exposed to Cr(VI) or not (see Supporting Information, page S14 for a detailed description of experimental details on sample preparation for TEM). Note that the TEM pictures demonstrated cell lysis following exposure to Cr(VI) compared to the control with no Cr(VI) exposure. It is also obvious that exposure to Cr(VI) not only changed the shapes and sizes of the cells, but also caused acute damage that eventually led to cell death.

Environmental Implications. The overall results indicate that microbial exudates play an important role on microbial Cr(VI) reduction due to the formation of soluble Cr-ligand complexes that are stable for extended periods of time in the environment.[13] These complexes protect the cells and Cr(VI) reductase enzymes from inactivation. Overall, this study highlights the need to consider the Cr−EPS interactions when assessing microbial Cr(VI) reduction and chromium transport in subsurface systems, including microbial reactive treatment barriers.

■ ASSOCIATED CONTENT

Supporting Information. A detailed description of soil properties, batch, and column experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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